Amino Acids (2013) 44:143–150
DOI 10.1007/s00726-011-1153-2

ORIGINAL ARTICLE

Probing the specificity of gamma-glutamylamine cyclotransferase: an enzyme involved in the metabolism of transglutaminase-catalyzed protein crosslinks

Todd E. Bowser · Mary Lynn Trawick

Received: 30 June 2011 / Accepted: 8 November 2011 / Published online: 27 November 2011

Abstract γ-Glutamylamine cyclotransferase (gGACT) catalyzes the intramolecular cyclization of a variety of L-γ-glutamylamines producing 5-oxo-L-proline and free amines. Its substrate specificity implicates it in the downstream metabolism of transglutaminase products, and is distinct from that of γ-glutamyl cyclotransferase which acts on L-γ-glutamyl amino acids. To elucidate the mechanism by which gGACT distinguishes between L-γ-glutamylamine and amino acid substrates, the specificity of the rabbit kidney enzyme for the amide region of substrates was probed through the kinetic analysis of a series of L-γ-glutamylamines. The isodipeptide N^{ε}-(L-γ-glutamyl)-L-lysine **1** was used as a reference. The kinetic constants of the L-γ-glutamyl derivative of *n*-butylamine **7**, were nearly identical to those of **1**. Introduction of a methyl or carboxylate group on the carbon adjacent to the side-chain amide nitrogen in L-γ-glutamylamine substrates resulted in a dramatic decrease in substrate properties for gGACT thus providing an explanation of why gGACT does not act on L-γ-glutamyl amino acids except for L-γ-glutamylglycine. Placement of substituents on carbons further removed from the side-chain amide nitrogen in L-γ-glutamylamines restored activity for gGACT, and L-γ-glutamylneohexylamine **19** had a higher specificity constant (k_{cat}/K_m) than **1**. gGACT did not exhibit any stereospecificity in the amide region of L-γ-glutamylamine substrates. In addition, analogues (**26**–**30**) with heteroatom substitutions for the γ methylene position of the L-γ-glutamyl moiety were examined. Several thiocarbamoyl derivatives of L-cysteine (**28**–**30**) were excellent substrates for gGACT.

Keywords Gamma-glutamylamine cyclotransferase · Gamma-glutamyl cyclotransferase · Transglutaminase · L-γ-glutamylamines · *S*-(*n*-butylcarbamyl)-L-cysteine · *O*-(*n*-butylcarbamyl)-L-serine

Introduction

γ-Glutamylamine cyclotransferase (gGACT) (Danson et al. 2002; Fink et al. 1980; Fink and Folk 1981a, b, 1983), a cytosolic enzyme widely distributed in animal cells and tissues, catalyzes the release of free amines from L-γ-glutamylamines with the concomitant intramolecular cyclization of the L-γ-glutamic acid moiety to 5-oxo-L-proline as shown in Fig. 1. Among its substrates are N^{ε}-(L-γ-glutamyl)-L-lysine, L-γ-glutamylhistamine, and both the mono- and di-L-γ-glutamyl derivatives of putrescine, spermidine, and spermine. The specificity of gGACT for these substrates indicates that the enzyme functions in the downstream metabolism of proteins crosslinked through N^{ε}-(L-γ-glutamyl)-L-lysine linkages and other protein products of transglutaminase catalyzed-action (Fink and Folk 1981a, b) (Fig. 1). Transglutaminases (Tgases) are a family of enzymes that are structurally and functionally related and are implicated in many cellular processes including stabilization of blood clots, wound healing, cell differentiation,

Dedicated to the late Dr. John E. Folk, who synthesized many of the compounds used in this study, for his long-standing collaboration, and his unfailing encouragement.
Mary Lynn Trawick was formerly Mary Lynn Fink.

Electronic supplementary material The online version of this article (doi:10.1007/s00726-011-1153-2) contains supplementary material, which is available to authorized users.

T. E. Bowser · M. L. Trawick (✉)
Department of Chemistry and Biochemistry and the Institute of Biomedical Studies, Baylor University, Waco, TX 76798, USA
e-mail: Mary_Lynn_Trawick@baylor.edu

apoptosis, autophagy, and inflammatory diseases such as Celiac disease (Beninati et al. 2009; Davies et al. 1985; Fesus et al. 1991; Folk 1980; Folk and Finlayson 1977; Facchiano and Facchiano 2009). Tgases catalyze protein crosslinking through N^{ε}-(L-γ-glutamyl)-L-lysine linkages, and the incorporation of amines including histamine, and the polyamines into proteins (Beninati et al. 1985; Folk 1980; Folk and Finlayson 1977; Folk et al. 1980).

gGACT was first isolated by its ability to readily catalyze the release of L-lysine from the proteolytically resistant isodipeptide, N^{ε}-(L-γ-glutamyl)-L-lysine **1** (Fink et al. 1980). Recently, the gene for gGACT was identified, cloned, and the X-ray crystal structure of the expressed protein was determined (Oakley et al. 2010). The human gGACT protein is a homodimer of 21 kD with a mixed α/β topology and an essential active-site Glu^{82} residue that promotes acid–base catalysis (Oakley et al. 2010).

Previous substrate specificity studies of gGACT tested the activity of various L-γ-glutamylamines and other

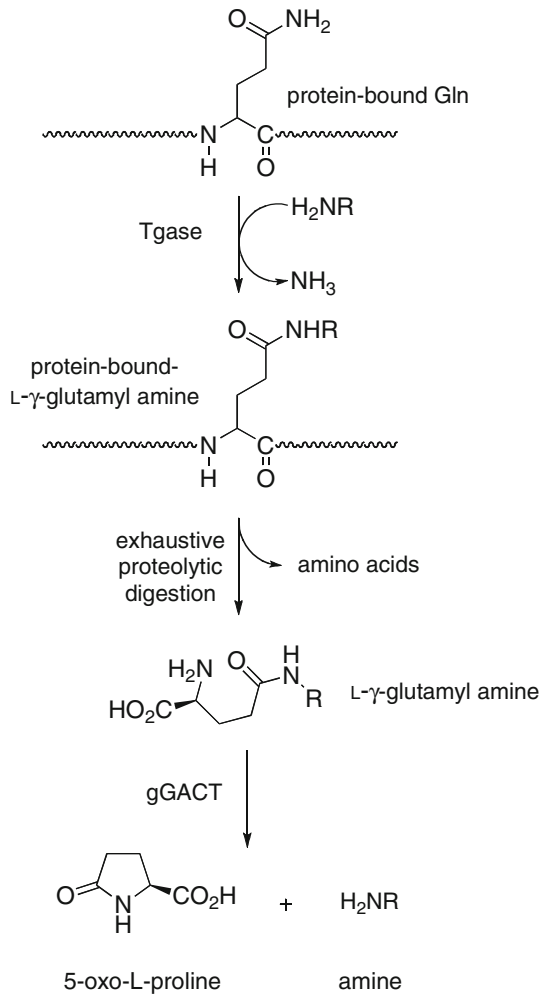


Fig. 1 Proposed metabolism of transglutaminase (Tgase)-catalyzed protein crosslinks and protein-bound γ-glutamylamines

L-γ-glutamylamine analogs relative to the known substrate, N^{ε}-(L-γ-glutamyl)-L-lysine (Fink et al. 1980; Fink and Folk 1981a). These studies demonstrated that gGACT has a stringent requirement for the peptide-free L-γ-glutamyl portion of the substrate, since compounds in which the α-amino or α-carboxyl group of the glutamic acid moiety was blocked failed to show activity as substrates. Although the enzyme displayed high specificity for the L-γ-glutamyl portion of the substrate, the specificity for the side-chain amide portion of the substrate was much broader. Also, N^{ε}-(L-γ-glutamyl)-L-lysine analogs in which the L-lysine portion was replaced by α-*N*-acetyl-L-lysine-methyl ester or D-lysine were good substrates. Although the enzyme displayed wide specificity for L-γ-glutamylamines, important exceptions were noted. For example, α-(L-γ-glutamyl)-L-amino acids and L-γ-glutamyl peptides such as γ-glutamylglycyl-β-alanine were not substrates for gGACT (Fink et al. 1980; Fink and Folk 1981a). Preliminary studies indicated pronounced variations in the activity of L-γ-glutamylamines as substrates. This preference and the failure of α-(L-γ-glutamyl)-L-amino acids to act as substrates is consistent with the proposal that gGACT is a distinct enzyme from γ-glutamyl cyclotransferase (gGCT), which catalyzes the cyclization of α-(L-γ-glutamyl)-L-amino acids producing 5-oxo-L-proline and a free amino acid (Orlowski and Meister 1973; Orlowski et al. 1969; Oakley et al. 2008). To investigate the detailed substrate specificity of gGACT, the kinetic analysis of a series of L-γ-glutamylamines and L-γ-glutamylamine analogues was carried out. Particularly, we were interested in probing the side-chain amide region specificity of gGACT in order to understand the mechanism by which the enzyme is capable of distinguishing α-(L-γ-glutamyl)-L-amino acids from N^{ε}-(L-γ-glutamyl)-L-lysine and various other L-γ-glutamylamines. Kinetic analysis of a number of L-γ-glutamyl amino acids and L-γ-glutamylalkylamines, including the new compounds, the L-γ-glutamylamines of 2-methylbutlyamine **15**–**16**, *sec*-butylamine **11**–**12**, neopentylamine **18**, and neohexylamine **19**, were carried out to explore the effects of chain length, stereochemistry, branching, steric interactions, and polarity on the amide region specificity of the enzyme. In addition, the effect of substituting the γ-carbon of the L-glutamyl moiety with oxygen, sulfur, or nitrogen was tested using the kinetic analysis of a number of L-γ-glutamylalkylamine analogues of cysteine, serine, and urea, including the new compound *O*-(*n*-butylcarbamyl)-L-serine **27**. This report provides strong evidence for the specificity of gGACT for the amide region of L-γ-glutamylamines and the mechanism whereby the enzyme distinguishes various L-γ-glutamylamines and L-γ-glutamyl amino acids. This study also demonstrates the unexpected high specificity of the enzyme for cysteine analogues of L-γ-glutamylamines providing insight into the

steric and electronic effects of the sulfur substitution at the γ-position of the L-γ-glutamyl region of the substrate.

Experimental

Experimental details for the synthesis and characterization of compounds **1–30**, which were prepared by procedures described in the literature, are given in Supplementary Material. Information on enzyme assays, the purification of gGACT from rabbit kidney, and its separation from γ-glutamyl cyclotransferase is also available in Supplemental Material.

Results and discussion

Kinetic analysis

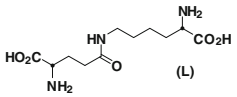
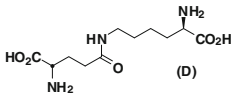
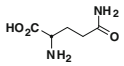
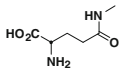
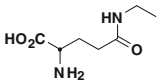
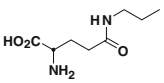
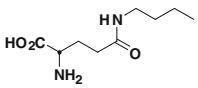
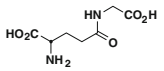
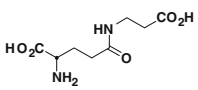
To elaborate the substrate specificity of gGACT and to understand the mechanism by which the enzyme discriminates between various L-γ-glutamylamines and L-γ-glutamylamino acids, a series of compounds were synthesized and tested as substrates for gGACT, and the kinetic parameters, the catalytic constant k_{cat}, the K_m, and the specificity constant k_{cat}/K_m were determined for each. Issues of stereochemistry, carboxylate placement, and branching in the amide region of substrates were examined in addition to the substitution of heteroatoms for the gamma carbon in the L-γ-glutamyl region of substrates. In contrast to the stringent requirement for an L-γ-glutamyl moiety in substrates, no stereochemical requirement was observed in the lysine portion of substrates (Table 1). Both N^{ε}-(L-γ-glutamyl)-L-lysine **1** ($k_{cat} = 0.20 \pm 0.01$ s^{-1}, $K_m = 0.26 \pm 0.02$ mM, $k_{cat}/K_m = 0.78 \pm 0.05$ mM^{-1} s^{-1}) and N^{ε}-(L-γ-glutamyl)-D-lysine **2** ($k_{cat} = 0.25 \pm 0.01$ s^{-1}, $K_m = 0.27 \pm 0.03$ mM, $k_{cat}/K_m = 0.92 \pm 0.06$ mM^{-1} s^{-1}), which differ only in stereochemistry at the α-carbon of lysine, functioned as comparable substrates for gGACT. Although L-glutamine **3** was not a substrate for gGACT, in general the catalytic rate constant (k_{cat}) values increased as the side-chain amide increased in length proceeding, from L-γ-glutamyl-*n*-methylamine **4** ($k_{cat} = 0.02$ s^{-1}), L-γ-glutamyl-*n*-ethylamine **5** ($k_{cat} = 0.038 \pm 0.001$ s^{-1}), to L-γ-glutamyl-*n*-propylamine **6** ($k_{cat} = 0.23 \pm 0.001$ s^{-1}, Table 1). In this series, the K_m values decreased resulting in a progressive increase in the corresponding specificity constants. The kinetic constants obtained for L-γ-glutamyl-*n*-butylamine **7** ($k_{cat} = 0.21 \pm 0.002$ s^{-1}, $K_m = 0.25 \pm 0.01$ mM, $k_{cat}/K_m = 0.83 \pm 0.01$ $mM^{-1}s^{-1}$, Table 1) were nearly identical to the values obtained for N^{ε}-(L-γ-glutamyl)-L-lysine **1**. This indicates that, even though gGACT shows little specificity for the α-carbon of lysine, the enzyme does display specificity for a four carbon, alkyl side-chain and shortening of this side-chain results in a significant loss of activity.

γ-Glutamyl cyclotransferase, which uses γ-glutamylamino acids as substrates, and gGACT are distinct enzymes, and gGACT does not act on α-(L-γ-glutamyl)-L-lysine, L-γ-glutamyl-L-methionine, or L-γ-glutamyl-L-glutamine (Fink and Folk 1981a). However L-γ-glutamylglycine **8** is a substrate, albeit a poor one ($k_{cat} = 0.14 \pm 0.02$ s^{-1}, $K_m = 1.9 \pm 0.6$ mM, $k_{cat}/K_m = 0.071 \pm 0.009$ mM^{-1} s^{-1}, Table 1). Location of the carboxyl group on carbon two of the amide side-chain as in L-γ-glutamyl-β-alanine **9** significantly improves the K_m value to 0.91 ± 0.06 mM and therefore the specificity constant (0.19 ± 0.01, Table 1). This indicates that placement of a carboxyl group is an important factor, but not the only consideration in the ability of gGACT to discriminate between L-γ-glutamylamines and L-γ-glutamyl amino acids. The idea that branching in the amide region of substrates might play a role was investigated with a series of compounds **10–19** shown in Table 2.

Placement of a methyl branch on the carbon adjacent to the side-chain amide nitrogen resulted in a significant decrease in k_{cat} values to 0.11 s^{-1} or lower for substrates **10–14** (Table 2) in comparison to substrates with unbranched side-chains (Table 1). In general, the K_m values were higher (1.62 ± 0.21 mM for **11**, for example) than that of N^{ε}-(L-γ-glutamyl)-L-lysine **1** with the exception of L-γ-glutamyl-(±)-3-aminobutyric acid **13** ($K_m = 0.14 \pm 0.02$ mM, Table 2). The activity measured for the highly substituted L-γ-glutamyl-*t*-butylamine **14** was 2.3 μmol product/mg of enzyme/h. The combination of the very low activity for **14** and the slow reaction of product *t*-butylamine with *o*-phthalaldehyde made the fluorescent detection of *t*-butylamine difficult. Therefore, we were unable to obtain reliable kinetic constants for **14**. This low activity and the trend for compounds **10–14** (Table 2) is consistent with the proposal that branching at the first carbon adjacent to the amide nitrogen position introduces unfavorable steric interactions between the enzyme and substrates. It should be noted that both the *S* isomer **11** and the *R/S* mixture **12** of L-γ-glutamyl-*sec*-butylamine gave comparable kinetic values indicating no stereochemical preference at this position.

Methyl branching at the second carbon of the amide side-chain restored considerable activity as seen in the L-γ-glutamyl-2-methylbutylamine substrates **15–16**. Their k_{cat} values were comparable to each other and to that of **1** although their K_m values were higher (0.38 mM versus 0.26 mM). Again the *S* isomer **15** and the *R/S* mixture **16** of L-γ-glutamyl-2-methylbutylamine gave comparable kinetic values indicating no stereochemical preference in the

Table 1 Effect of stereochemistry, chain length, and carboxyl placement in the amide region of substrates

Substrate		k_{cat} $(s)^{-1}$ (±SE)	K_m (mM) (±SE)	k_{cat}/K_m $(mM\ s)^{-1}$ (±SE)
	**1**	0.20 (±0.01)	0.26 (±0.02)	0.78 (±0.05)
	**2**	0.25 (±0.01)	0.27 (±0.03)	0.92 (±0.06)
	**3**	n.a.[a]	n.d.	n.d.
	**4**	0.02[a]	n.d.	n.d.
	**5**	0.038 (±0.001)	0.36 (±0.02)	0.11 (±0.01)
	**6**	0.23 (±0.01)	0.32 (±0.05)	0.72 (±0.05)
	**7**	0.21 (±0.002)	0.25 (±0.01)	0.83 (±0.01)
	**8**	0.14 (±0.02)	1.9 (±0.6)	0.071 (±0.009)
	**9**	0.18 (±0.01)	0.91 (±0.06)	0.19 (±0.01)

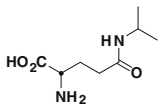
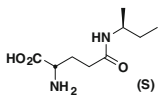
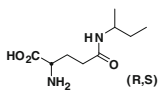
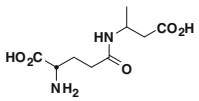
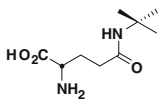
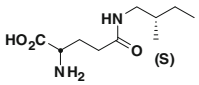
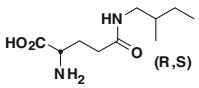
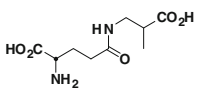
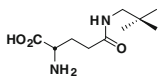
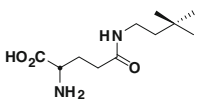
n.a. not active, *n.d.* not determined

[a] Fink and Folk 1981a

amide side-chain of substrates. L-γ-Glutamyl-(±)-3-aminoisobutyric acid **17** had a similar K_m (0.33 ± 0.04 mM, Table 2) to those of **15** and **16**, but a much lower k_{cat} value (0.071 ± 0.002 s^{-1}). Substitution of two methyl groups at the second position, as in L-γ-glutamylneopentylamine **18**, resulted in a dramatic loss of activity (k_{cat} = 0.035 ± 0.001 s^{-1}, K_m = 0.61 ± 0.05 mM, k_{cat}/K_m = 0.057 ± 0.007 $mM^{-1}s^{-1}$) relative to **15** and **16**. However, L-γ-glutamylneohexylamine **19** in which the quaternary carbon is moved to the third carbon of the amide side-chain was an excellent substrate (k_{cat} = 0.18 ± 0.003 s^{-1}, K_m = 0.12 ± 0.01 mM, k_{cat}/K_m = 1.45 ± 0.05). These results underscore the importance of steric interaction and substituent position on the amide side-chain of L-γ-glutamyl substrates. This concept was further explored in a series of substrates **20**–**25** containing ring structures.

L-γ-Glutamylamines with cycloalkane structures substituted on the amide side-chains **20**–**22**, and L-γ-glutamylaniline **23** exhibited low catalytic constants (Table 3) similar to those of substrates with methyl branching at the carbon adjacent to the amide nitrogen (Table 2). Interestingly, several of these compounds such as L-γ-glutamylcyclopentylamine **21** and L-γ-glutamylaniline **23** also had low K_m values. This suggests a contribution from a non-productive binding mode which characteristically serves to decrease both the k_{cat} and K_m, while leaving k_{cat}/K_m unaffected. This may also be the case with L-γ-glutamyl-(±)-3-aminobutyric acid **13** (K_m = 0.14 ± 0.02 mM, Table 2). One possible mode of non-productive binding could occur if the compound binds in a backwards sense to the productive mode wherein the amide side-chain of the compound binds to the enzyme at the location where L-γ-glutamyl moiety of the substrate would bind in order for the reaction to take place. The transition state of the gGACT catalyzed reaction with L-γ-glutamylamines is expected to be close to the geometry and electronic configuration of the five-membered cyclic intermediate. In that respect, it is interesting

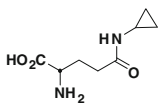
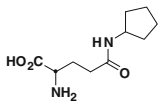
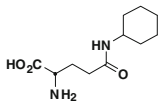
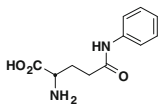
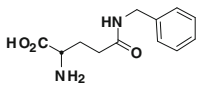
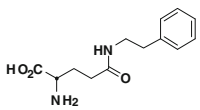
Table 2 Effect of branching in the amide region of substrates

Substrate		k_{cat} $(s)^{-1}$ (±SE)	K_m (mM) (±SE)	k_{cat}/K_m $(mM\ s)^{-1}$ (±SE)
	**10**	0.068 (±0.004)	0.69 (±0.09)	0.097 (±0.008)
	**11**	0.11 (±0.005)	1.62 (±0.21)	0.066 (±0.006)
	**12**	0.07 (±0.002)	1.24 (±0.13)	0.058 (±0.005)
	**13**	0.054 (±0.003)	0.14 (±0.02)	0.39 (±0.04)
	**14**	<0.012	n.d.	n.d.
	**15**	0.21 (±0.02)	0.38 (±0.07)	0.55 (±0.06)
	**16**	0.19 (±0.01)	0.38 (±0.06)	0.51 (±0.05)
	**17**	0.071 (±0.002)	0.33 (±0.04)	0.21 (±0.02)
	**18**	0.035 (±0.001)	0.61 (±0.05)	0.057 (±0.007)
	**19**	0.18 (±0.003)	0.12 (±0.01)	1.45 (±0.05)

n.d. not determined

to note that L-γ-glutamylcyclopentylamine **21** had the lowest K_m of any of the substrates in this study. Moving a benzene ring one methylene group away from the amide nitrogen yields L-γ-glutamylbenzylamine **24** (Table 3) which exhibited a k_{cat} of 0.40 ± 0.03 s^{-1} and a K_m of 0.56 ± 0.08 mM. This correlates to a two-fold increase in the specificity constant relative to L-γ-glutamylaniline **23**. Movement of the aromatic ring to the second carbon in the amide side-chain position significantly relieves steric hindrance associated with the branching at the first amide position (as in the aniline derivative **23**), and yields a side-chain of almost equal length for **24** (≈4.88 Å as determined by ChemDraw 3D) to the *n*-butylamine side-chain (≈4.92 Å) in **7**. Moving the benzene ring a second methylene group further from the amide yields L-γ-glutamyl-β-phenethylamine **25** (Table 3). The k_{cat} of 0.17 ± 0.01 s^{-1} and the K_m of 0.44 ± 0.05 mM determined for **25** translate to a k_{cat}/K_m about one-half the value obtained for L-γ-glutamylbenzylamine. This may indicate that, even though **25** contains no branching at the first or second position of the amide side-chain, the non-polar β-phenethylamine group may extend beyond a hydrophobic pocket in the active-site of the enzyme into a hydrophilic region. The extended β-phenethylamine group in **25** extends approximately 6.20 Å compared to the approximation of 4.92 Å for the *n*-butylamine side-chain of **7**. This proposal is consistent with the kinetic data obtained for the lysine derivatives **1** and **2**. Although gGACT apparently

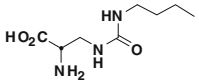
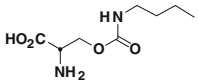
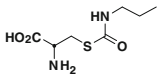
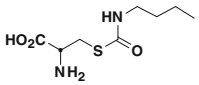
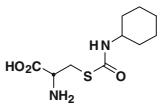
Table 3 Effect of ring structures in the amide region of substrates

Substrate		k_{cat} $(s)^{-1}$ (±SE)	K_m (mM) (±SE)	k_{cat}/K_m $(mM\ s)^{-1}$ (±SE)
	**20**	0.045 (±0.002)	0.22 (±0.02)	0.21 (±0.01)
	**21**	0.076 (±0.001)	0.084 (±0.004)	0.90 (±0.03)
	**22**	0.061 (±0.001)	0.32 (±0.02)	0.19 (±0.01)
	**23**	0.036 (±0.001)	0.10 (±0.01)	0.35 (±0.02)
	**24**	0.40 (±0.03)	0.56 (±0.08)	0.71 (±0.05)
	**25**	0.17 (±0.01)	0.44 (±0.053)	0.38 (±0.04)

showed little if any specificity for the α-amino and α-carboxyl of lysine in these substrates, these polar groups were still tolerated without a loss in specificity which suggests the presence of a hydrophilic enzyme region beyond the area that binds the four carbons of the lysine side-chain.

Finally, the substitution of heteroatoms for the gamma methylene position of the L-γ-glutamyl region of substrates was examined (Table 4). The substitution of a nitrogen for the γ-methylene carbon (L-2-amino-3-(3-*n*-butylureido)-propionic acid **26**) abolished activity as a substrate within detection limits (the detectable limit of the assay procedure was 0.13 μmol of *n*-butylamine/mg of enzyme/h). These results suggest that the urea functionality may not be susceptible to nucleophilic attack by the α-amino group due to the high delocalization energy of the urea. Also, the bond lengths, bond angles, and polarity of the nitrogen, as compared to the methylene group of L-γ-glutamylamine substrates may introduce unfavorable electronic interactions or cause a poor fit of the compound within the active-site of the enzyme. *O*-(*n*-Butylcarbamyl)-L-serine **27** in which an oxygen replaces the methylene group exhibited by far the largest catalytic constant observed for any substrate tested. The observed k_{cat} value was 57-fold higher than the k_{cat} of L-γ-glutamyl-*n*-butylamine **7**. This large k_{cat} for **27** was accompanied by an even larger increase in K_m to 20 ± 1 mM (80-fold relative to the value of 0.25 ± 0.01 mM observed for L-γ-glutamyl-*n*-butylamine **7**) resulting in a k_{cat}/K_m value for **27** of only 0.60 ± 0.06 $mM^{-1}\ s^{-1}$. The high K_m obtained for the serine analog suggests that it is most likely not a physiological substrate for the enzyme. It also suggests that the substitution of the polar oxygen for the non-polar methylene introduces unfavorable electronic interactions with hydrophobic active-site side-chains. The k_{cat} value for *O*-(*n*-butylcarbamyl)-L-serine (**27**) is somewhat puzzling, since it has been shown that there is a significant amount of delocalization in the carbamate functionality (ROC(O)NHR) (Ferraz de Souza et al. 1996; Waszkowycz et al. 1991) which should make the nucleophilic attack on the carbamate carbonyl carbon at least as difficult as the attack on the amide carbonyl of the L-γ-glutamylamines. The large reaction rate observed for the carbamate, *O*-(*n*-butylcarbamyl)-L-serine **27**, in this study suggests that the binding interaction of **27** with the enzyme active-site may destabilize the delocalization energy of the carbamate. This may occur through torsion of the carbon to amide nitrogen or carbon to ester oxygen bond or through the electronic effects of hydrogen bonding or electrostatic bonding to the carbamate functionality by active-site residues. A loss in delocalization energy within the carbamate could be paid

Table 4 Modification of the glutamyl region in substrates

Substrate		k_{cat} $(s)^{-1}$ (±SE)	K_m (mM) (±SE)	k_{cat}/K_m $(mM\ s)^{-1}$ (±SE)
	**26**	<0.001	n.d.	n.d.
	**27**	12.0 (±0.3)	20.0 (±1.0)	0.60 (±0.06)
	**28**	0.24 (±0.01)	0.093 (±0.009)	2.6 (±0.2)
	**29**	0.27 (±0.01)	0.091 (±0.007)	2.8 (±0.1)
	**30**	0.26 (±0.01)	0.19 (±0.02)	1.5 (±0.8)

n.d. not determined

for by the favorable binding interaction of the substrate and enzyme.

Cysteine analogues of L-γ-glutamylamines are excellent substrates for gGACT (Table 4). The k_{cat}/K_m determined for *S*-(*n*-propylcarbamyl)-L-cysteine **28** (Table 4) with gGACT was 2.6 ± 0.2 $mM^{-1}\ s^{-1}$ for gGACT, and *S*-(*n*-butylcarbamyl)-L-cysteine **29** was the best substrate of this group of compounds (k_{cat} = 0.27 ± 0.01 s^{-1}, K_m = 0.091 ± 0.007 mM and k_{cat}/K_m = 2.8 ± 0.1 $mM^{-1}\ s^{-1}$). The substitution of the methylene group with the larger, more polarizable sulfur atom apparently enhances a favorable interaction with the enzyme through non-polar interactions or increasing the effective ring size of the cyclic transition state through its longer carbon to sulfur bonds which could enhance substrate interaction with the active-site side-chains. Also, the significant delocalization of the thiocarbamate functionality (Ferraz de Souza et al. 1996) could serve to decrease rotational freedom of the substrate and enhance the interaction of the enzyme with the sulfur analogs. Replacing the *n*-butylamine side-chain of **29** with cyclohexylamine as in *S*-(cyclohexylamine)-L-cysteine **30** (Table 4), resulted in an increase in the observed K_m value to 0.19 ± 0.02 mM, but the k_{cat} value was unaffected. This is in contrast to the L-γ-glutamyl analogues which resulted in a decrease in the k_{cat} value of over 70% with the corresponding change in the substrate side-chain (**7** and **22**, Tables 1, 3). This lowered ability to distinguish between the various amide side-chains of L-cysteine analogues (possibly due to a slightly different, but productive mode of binding of the L-cysteinyl moiety of these substrates) would argue against *S*-carbamoylated cysteine compounds as substrates for gGACT, but their physiological occurrence is unknown and requires further investigation.

The results presented in this study suggest that gGACT contains a narrow, hydrophobic pocket which conveys specificity for L-γ-glutamylamines with unbranched, alkyl amides extending approximately four carbons or 4.92 Å. Beyond this pocket the enzyme may have little interaction with substrates since L-γ-glutamyl-*n*-butylamine **7** exhibited the same kinetic constants as the L-γ-glutamyl derivatives of L- and D-lysine (**1** and **2**, respectively). However, compounds with alkyl chains that extended less than four carbons exhibited significant decreases in activity and k_{cat}/K_m values. A hydrophobic pocket of gGACT appears to open as it continues down the amide moiety of the substrate, since branching at the first amide carbon was associated with low k_{cat} values (**10**–**14**, **20**–**23**), yet a single branch at the second amide carbon was more readily accommodated and branching at the third carbon resulted in a lower K_m and an excellent specificity constant for **19**. These results are consistent with a recently published X-ray crystal structure for gGACT which indicates that the active-site cavity has a region that is narrower in gGACT compared with γ-glutamyl cyclotransferase (Oakley et al. 2010). Binding of gGACT to substrates is especially sensitive to steric hindrance at the carbon adjacent to the amide nitrogen and the presence of polar carboxyl groups at this position. These results show that a combination of branching and carboxylate substitution at this position accounts for the lack of activity of gGACT toward L-γ-glutamylamino acids with the exception of L-γ-glutamylglycine.

These results not only lend support to the proposal that gGACT functions in the catabolism of transglutaminase products, but also provide evidence for the design of potent active-site directed inhibitors by combining the lessons learned from the amide specificity and heteroatom substitutions at the γ-methylene position of L-γ-glutamylamines.

Acknowledgments The authors are grateful for the contribution of the late Dr. John E. Folk who synthesized many of the compounds used in this study. We express our appreciation to Mr. Gustavo E. Chavarria for expert assistance in the preparation of this manuscript. This work was supported by the University Research Committee, Baylor University.

References

Beninati S, Bergamini CM, Piacentini M (2009) An overview of the first 50 years of transglutaminase research. Amino Acids 36:591–598

Beninati S, Piacentini M, Argento-Cerù MP, Russo-Caia S, Autuori F (1985) Presence of di- and polyamines covalently bound to protein in rat liver. Biochim Biophys Acta 841(1):120–126

Danson JW, Trawick ML, Cooper AJL (2002) Spectrophotometric assays for L-lysine alpha-oxidase and gamma-glutamylamine cyclotransferase. Anal Biochem 303:120–130

Davies PJ, Murtaugh MP, Moore WT Jr, Johnson GS, Lucas D (1985) Retinoic acid-induced expression of tissue transglutaminase in human promyelocytic leukemia (HL-60) cells. J Biol Chem 260:5166–5174

Ferraz de Souza W, Kambe N, Sonoda N (1996) Theoretical study of structures and internal rotations of methyl N, N-dimethylcarbamate and its sulfur, selenium, and tellurium homologs (Me_2N-C(O)YMe, Y=O, S, Se, Te). J Phys Org Chem 9(3):179–186

Facchiano A, Facchiano F (2009) Transglutaminases and their substrates in biology and human diseases: 50 years of growing. Amino Acids 36:599–614

Fesus L, Davies PJ, Piacentini M (1991) Apoptosis: molecular mechanisms in programmed cell death. Eur J Cell Biol 56(2):170–177

Fink ML, Chung SI, Folk JE (1980) gamma-Glutamylamine cyclotransferase: specificity toward epsilon-(L-gamma-glutamyl)-L-lysine and related compounds. Proc Natl Acad Sci USA 77:4564–4568

Fink ML, Folk JE (1981a) γ-Glutamylamine cyclotransferase: an enzyme involved in the catabolism of ε-(γ-glutamyl)lysine and other γ-glutamylamines. Mol Cell Biochem 38:59–67

Fink ML, Folk JE (1981b) γ-Glutamylpolyamines: implications in the degradation of polyamine-protein conjugates. In Caldarera CM et al (eds) Advances in polyamine research, vol 3. Raven Press, New York, pp 187–196

Fink ML, Folk JE (1983) gamma-Glutamylamine cyclotransferase (rabbit kidney). In: Tabor H, Tabor CW (eds) Polyamines. Methods enzymology, vol 94. Academic Press, New York, pp 347–351

Folk JE, Finlayson JS (1977) The epsilon-(gamma-glutamyl)lysine crosslink and the catalytic role of transglutaminases. Adv Protein Chem 31:1–133

Folk JE (1980) Transglutaminases. Ann Rev Biochem 49:517–531

Folk JE, Park MH, Chung SI, Schrode J, Lester EP, Cooper HL (1980) Polyamines as physiological substrates for transglutaminases. J Biol Chem 255:3695–3700

Oakley AJ, Yamada T, Liu D, Coggan M, Clark AG, Board PG (2008) The identification and structural characterization of C7orf24 as γ-glutamyl cyclotransferase: an essential enzyme in the γ-glutamyl cycle. J Biol Chem 283:22031–22042

Oakley AJ, Coggan M, Clark AG, Board PG (2010) Identification and characterization of γ-glutamylamine cyclotransferase, an enzyme responsible for γ-glutamyl-ε-lysine catabolism. J Biol Chem 285:9642–9648

Orlowski M, Meister A (1973) The γ-glutamyl cyclotransferase. Distribution, isoenzymic forms, and specificity. J Biol Chem 248(8):2836–2844

Orlowski M, Richman PG, Meister A (1969) Isolation and properties of γ-L-glutamylcyclotransferase from human brain. Biochemistry 8(3):1048–1055

Waszkowycz B, Hillier IH, Gensmantel N, Payling DW (1991) Combined quantum mechanical-molecular mechanical study of catalysis by the enzyme phospholipase A2: an investigation of the potential energy surface for amide hydrolysis. J Chem Soc Perkin Trans 2; Phys Org Chem (1972–1999) (12):2025–2032